AD_____

GRANT NUMBER DAMD17-94-J-4118

TITLE: Biologic Effects of HER-2/Neu Gene Overexpression and Agonists and Antagonists to the Receptor in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Dennis J. Slamon, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, California 90024-1406

REPORT DATE: August 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1996	Annual (11 Jul 95 - 10 Jul 96)

4. TITLE AND SUBTITLE
Biologic Effects of HER-2/Neu Gene Overexpression and Agonists and Antagonists to the Receptor in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4118

6. AUTHOR(S)
Dennis J. Slamon, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Los Angeles, California 90024-1406

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 2170-25012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The alteration of the HER-2/*neu* gene has been shown to correlate with a poor prognosis in those patients whose tumors contain it. This has led to studies from our laboratory indicate that it may play a role in the pathogenesis of the disease for some patients. Given that the HER-2/*neu* gene encodes a growth factor receptor found on the membrane of tumor cells and given its potential role in the pathogenesis of some human breast cancers, it is a logical target for the development of new therapeutic approaches directed at this alteration. Studies with monoclonal antibodies directed against the extra cellular domain of the receptor indicate that many may have significant growth inhibitory properties. Recently ligands have been identified which interact either directly or indirectly with the human HER-2/*neu* receptor, however little is known about the biologic effects of these molecules. There is some controversy as to whether the ligands mediate growth stimulatory or growth inhibitory effects or both. A greater understanding about the biologic effects of HER-2/*neu* overexpression as well as the impact of agonists and antagonists to the receptor will be required to fully therapeutically exploit this gene alteration in human breast cancer. Finally, little is know about the biologic effects of other molecular alterations which may occur in combination with HER-2/*neu* expression.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	15
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

___ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature Date

Annual Report Table of Contents

DAMD17-94-J-4118

Page

PROGRESS REPORT

DAMD17-94-J-4118

BIOLOGIC EFFECTS OF HER-2/*neu* GENE OVEREXPRESSION AND AGONISTS AND ANTAGONISTS TO THE RECEPTOR IN HUMAN BREAST CANCER

PRINCIPAL INVESTIGATOR: Dennis J. Slamon, M.D., Ph.D.

The background and introduction to this Progress Report remain identical to the as in the initial proposal and are restated here in edited format.

INTRODUCTION

The HER-2/*neu* gene is amplified in 25-30% of human breast cancers. When the alteration was examined for association with clinical parameters, it was found that gene amplification was associated with a poor prognosis; i.e. those women whose tumor contained the alteration had a shorter disease-free and overall survival. This association was initially disputed, but a large number of subsequent studies have now confirmed it. Moreover, the prognostic association between HER-2/*neu* alteration and disease outcome is seen in both node positive and node negative breast cancer. This literature and the controversy surrounding the association has been extensively reviewed recently. The bulk of the published data now clearly support the prognostic significance of HER/*neu* overexpression in breast cancer. However, all of the reviewed studies were retrospective analyses of archival cohorts of specimens. Most recently the first prospective study, consisting of 1056 primary human breast cancers, was completed and published and this study also confirmed the prognostic significance of HER-2/*neu* alteration in both node negative and node positive disease . Taken together, these data confirm the prognostic significance of HER-2/*neu* amplification/overexpression in human breast cancer.

There are at least two possible explanations for the association between HER-2/*neu* amplification/overexpression and poor clinical outcome; a) the alteration serves as a genetic marker for a poor outcome but plays no role in causing it; i.e. a useful prognostic epiphenomenon; or alternatively b) the alteration is associated with a poor outcome because it plays a direct role in the pathogenesis of such an outcome. There is circumstantial evidence which supports the latter possibility. These data include the following: 1) the mutated rat *neu* gene can act as a dominant transforming oncogene, underscoring its oncogenic potential and NIH 3T3 cells transfected with the gene are not only transformed *in vitro*, but are tumorigenic in the nude mouse ; 2) monoclonal antibodies directed against the extracellular domain of the rat *neu* gene will inhibit transformation *in vitro* as well as tumorigenicity *in vivo*; 3) studies to develop transgenic mice using the mutated rat gene under the control of an MMTV promoter, demonstrate that these mice develop breast cancer at three months of age; and finally, 5) studies using a transfected human HER-2/*neu* gene in NIH 3T3 cells demonstrate

that it too will transform the cells and that higher levels of expression result in greater transforming efficiency, and greater tumorigenicity of the cells . In composite, these data clearly show the ability of the HER-2/*neu* gene to mediate transformation *in vitro* and tumorigenicity *in vivo*.

Each of these lines of circumstantial evidence, however, have shortcomings with regards to implicating the HER-2/*neu* gene in the pathogenesis of human breast cancer. The first line of evidence proved that the rat *neu* gene could be oncogenic; however, sequence analysis showed the gene to have a point mutation in the transmembrane domain. To date, no such mutation has been identified in the human HER-2/*neu* gene. Instead the alteration found in human breast cancer is amplification and overexpression of the normal gene. In addition, the mutated rat *neu* gene induces neuroglioblastomas and not breast cancer in the animals. The monoclonal antibody studies were equally convincing that the mutated rat gene could have a role in transformation of neural tissue but, again, these antisera were not directed against the human protein nor were they used in altering the phenotype of human breast carcinoma cells overexpressing a normal, non-mutated, human HER-2/*neu* gene. The transgenic mouse studies were particularly compelling in showing that alterations in the rat *neu* gene could result in the development of breast carcinoma but, again, the study had used the mutated rat gene (48). Lastly, the data demonstrating that an overexpressed, non-mutated human HER-2/*neu* gene could transform NIH 3T3 cells proved that the human gene was oncogenic *in vitro* , but the experiments again used NIH 3T3 cells, not human breast cancer or breast epithelial cells. In addition, the levels of overexpression in these studies were far in excess of what is seen in most human breast cancer specimens in nature. To circumvent some of the concerns raised with the experiments utilizing the rat *neu* gene or murine cells (NIH-3T3), we recently designed a series of experiments to introduce the human gene into human breast cancer cells as well as non-transformed immortalized and normal non-immortalized human breast epithelial cells. Important in the studies is to mimic as closely as possible the alteration seen in human tumors and then determine the biologic effects (if any) of this alteration. Central to these studies was the use of the human gene in human cells and the objective to hold the levels of overexpression at or below those seen in actual human tumors; i.e. not to exceed levels found in primary and/or metastatic tumors in nature. These studies have not yet been published but have now been completed as part of this grant support. They are presented as preliminary data in the current report. We feel that they demonstrate our ability to conduct the studies detailed in this application.

In addition we wish to examine the effects that an additional alteration, mutation of the p53 gene, may have on cells overexpressing the HER-2/*neu* gene. There is considerable evidence that the conversion of a normal cell into a malignant cell is a multistep process which may involve the alteration of more than one if not several genes. Mutation of the p53 gene is one of the most common genetic alterations found in human malignancies and is frequently found in breast cancer. The mutation rate in breast cancer is reported to be from a low of 14% to a high of 58% with the most frequently reported rate being between 25-30% of all cases. This incidence makes p53 alteration a potentially important mutation in the pathogenesis of human breast cancer. Similar to the data with HER-2/*neu*, this concept is circumstantially supported by data indicating that p53 mutation is associated with aggressive subtypes of the disease and/or a poor prognosis. The concept gains further support with experimental data

demonstrating that introduction of a wild type p53 gene into a human breast cancer cell line containing a mutant gene will suppress the transformed phenotype. Data are now accumulating which indicate that alterations in the p53 gene are frequently associated with HER-2/*neu* alterations . This combination of mutations may be very important in the pathogenesis of some human breast cancers. The HER-2/*neu* overexpressing cell lines already developed as well as those proposed to be developed as part of this application should be useful in addressing this issue.

BODY OF REPORT

Specific Aim I - To further develop a series of human breast epithelial and cancer cell lines containing defined alterations in expression of the human HER-2/*neu* gene.

This specific aim has largely been accomplished in the first nine-twelve months of funding for this project (11/94 - 7/95). The methods used to achieve the goals of this Specific Aim involve the introduction of a full length human HER-2/*neu* c-DNA into a series of human breast epithelial cell lines representing normal breast epithelial cells, immortalized but non-transformed breast epithelial cells and breast cancer cells. The cell lines detailed in the proposal, i.e. T47D, MDA-MB-231, MDA-MB-435, BT-20 and BT-483 have all been successfully transfected and engineered to overexpress the HER-2/*neu* gene. These transfectants have been characterized for stable HER-2/*neu* overexpression and all appear to have this feature (at least at 6-months of follow-up). The biologic characterization of these cells has been similar to those studies presented in the preliminary data in the initial proposal, i.e. ^{3}H-thymidene incorporation, cell growth (*in vitro)* anchorage independent growth and tumorigenicity. In all assays the data for the newly established engineered cells are similar to the data for the MCF-7, B5 and HBL-100 cell lines. DNA synthetic rate increases significantly as does cell growth, anchorage independent growth and tumorigenicity. These data are important in that they demonstrate that the biologic effects of HER-2/*neu* overexpression seen in the MCF-7, B5 and HBL-100 cell lines are not restricted to just those cell lines but can also be achieved in all of the breast cancer cell lines evaluated. These findings lend substantial credence to the concept that overexpression of the HER-2/*neu* gene plays an important pathogenic role in the aggressive biologic behavior of those cells and tumors which contain it. More recent studies using some of these cell lines has lead to insights into the clinical observation that human breast cancers which overexpress the HER-2/*neu* receptor tend to be estrogen receptor negative. Studies performed in our laboratory and, in part, supported by the grant, have demonstrated a potentially important direct interaction between activation of the HER-2/*neu* receptor and down regulation of the estrogen receptor.

Since the last progress report we have put a major effort into determining the effects of the HER-2/*neu* overexpression on chemotherapeutic drug sensitivity. This was done because retrospective data from two large clinical trials in breast cancer suggested an association between HER-2/*neu* overexpression and resistance to chemotherapy. Results from the Intergroup Study 0011 (Allred, *et al.*, 1992) and the International (Ludwig) Breast Cancer Study Group (Gusterson, *et al.*, 1992) led investigators to conclude that node-negative breast cancer patients whose tumors contain HER-2/*neu* overexpression have a less favorable prognosis due to a lack of response to adjuvant

cyclophosphamide (CPA), methotrexate (MTX), and 5-fluorouracil (5-FU) chemotherapy (CMF). In addition, in a study of 68 patients with advanced breast cancer, Wright and colleagues reported a shortened survival for patients with HER-2/*neu* overexpression who were treated with mitoxantrone despite the fact that response rates between HER-2/*neu*-overexpressing and non-overexpressing tumors were similar, 50% vs. 58%, respectively (Wright, *et al.*, 1992). A study of HER-2/*neu* overexpression in epithelial ovarian cancer demonstrated that patients whose tumors had the alteration were more likely to fail chemotherapy with CPA and carboplatin (CBDCA) (Felip, *et al.*, 1995). Conversely, in a clinical series reviewed by Klijn *et al.*, patients with metastatic breast cancer and amplification of the HER-2/*neu* gene had a superior response to CMF chemotherapy (75%) compared to patients without HER-2/*neu* amplified tumors (45%) and the median length of progression-free survival from the start of chemotherapy was superior in patients whose tumors exhibited amplification (Berns, *et al.*, 1995; Klijn, *et al.*, 1993). Recently, data from the Cancer and Leukemia Group-B demonstrated that node-positive breast cancer patients with HER-2/*neu* overexpression derived a benefit from doxorubicin (DOX)-based adjuvant chemotherapy which is dose-dependent indicating that HER-2/*neu* overexpression may be associated with an increased response to DOX (Muss, *et al.*, 1994). In composite, the clinical data to date are somewhat contradictory and do not adequately define what role, if any, HER-2/*neu* overexpression plays in chemotherapy response. Moreover, there is little experimental data to address this potentially important question. Only one study evaluating *in vitro* chemosensitivity in HER-2/*neu*-overexpressing breast carcinoma cells has been published and demonstrated no significant difference in response to either 5-FU or DOX in HER-2/*neu*-transfected MCF7 cells while HER-2 overexpression was associated with a 2 to 4-fold increase in resistance to cisplatin (CDDP)(Benz, *et al.*, 1992). *In vitro* studies of lung cancer cell lines demonstrated an association between HER-2/*neu* expression levels and intrinsic chemoresistance to six different chemotherapeutic drugs (Tsai, *et al.*, 1993), and transfection of HER-2/*neu* cDNA into one lung cancer cell line resulted in an increase in drug resistance (Tsai, *et al.*, 1995).

In an attempt to further define the effect of HER-2/*neu* overexpression on sensitivity to chemotherapeutic drugs in human breast cancers, we used the HER-2/*neu* engineered breast cancer cell lines: MCF7, MDA-MB-231, MDA-MB-435, and BT-20. Again, all of the parental cell lines used for this study contain a single copy of the HER-2/*neu* gene and express normal levels of the gene product while the matched HER-2/*neu* retroviral transfectants overexpress the gene. Dose-response curves using seven different classes of chemotherapeutic agents were constructed for the HER-2/*neu*-overexpressing cell lines as well as their mock-transfected parental controls. The rationale for this experimental approach was to allow direct comparison of genetically identical parent/daughter cells which differ only in that one member of the pair overexpresses the human HER-2/*neu* gene. This approach was taken to circumvent the difficulty of comparing cell lines derived from separate sources which may inherently differ in characteristics other than HER-2/*neu* overexpression which could impact on drug sensitivity. The rationale for evaluating more than one cell line representing this human malignancy is to avoid the possibility that any given observation could be unique to an individual cell line rather than being representative of a more generic biologic effect associated with HER-2/*neu* overexpression. Finally, to avoid the possibility that any observed effects might be restricted to an *in vitro* setting and because monolayer cell culture assays may not detect important multicellular mechanisms of drug

resistance chemosensitivity was tested *in vivo* for breast cancer parent/daughter xenografts in an athymic mouse model.

In vitro dose-response curves following exposure to 7 different classes of chemotherapeutic agents were compared for HER-2- and control-transfected cells. Chemosensitivity was also tested *in vivo* for HER-2- and control-transfected human breast cancer xenografts in athymic mice. These studies indicate that HER-2/*neu* overexpression was not sufficient to induce intrinsic, pleomorphic drug resistance. Furthermore, changes in chemosensitivity profiles resulting from HER-2/*neu* transfection observed *in vitro* were cell-line specific. *In vivo*, HER-2/*neu*-overexpressing breast cancer xenografts were responsive to different classes of chemotherapeutic drugs compared to control-treated xenografts with no statistically significant differences between HER-2/neu-overexpressing and non-overexpressing xenografts. We found no instance in which HER-2/*neu*-overexpressing xenografts were rendered more sensitive to chemotherapeutic drugs *in vivo*. HER-2/*neu*-overexpressing xenografts consistently exhibited more rapid regrowth than control xenografts following initial response to chemotherapy suggesting that a high rate of tumor cell proliferation rather than intrinsic drug resistance may be responsible for the adverse prognosis associated with HER-2/*neu* overexpression in human cancers.

Specific Aim II - To assess the biologic effects of agonists, i.e. the heregulin and *neu* differentiation factor ligands as well as an antagonist, i.e. a monoclonal antibody, to the HER-2/*neu* reception on human breast cells, *in vitro.*

The objective of this Specific Aim is to assess the biologic effects of HER-2/*neu* agonists and antagonists *in vitro*. The methods for this aim are detailed in the initial proposal and are unchanged. They will be mentioned in brief. The *in vitro* assays ^{3}H-thymidene incorporation, cell proliferation assays, clonogenic assays on plastic, anchorage independent growth assays (soft agar colony formation) and differentiation studies as determined by analysis of differentiation marker expression. In the past 12 months we have expanded and completed the ^{3}H-thymidene assay, cell proliferation assays, clonogenic assays and soft agar assays for the MCF-7, SKBr3, MDA-MB-231, MDA-MB-435, and BT-20 cells using the heregulin agonist and the 4D5 antagonist. The rationale for addressing this issue is the potentially critical role heregulin (the growth factor ligand for HER-2/neu) may play in human breast cancer. In the past four years, several putative ligands for p185$^{HER-2/neu}$ receptor have been described. Candidate ligands have been isolated from macrophages (Tarahovski et al., 1991), bovine kidney (Huang and Huang, 1992), conditioned medium from transformed human T cells (Dobashi et al, 1991), and rat transformed fibroblasts (Yarden and Peles, 1991), as well as from human breast cancer cells (Lupu et al, 1992a; Lupu et al, 1992b). However, none of the proteins described in those initial studies were isolated, purified to homogeneity, sequenced and/or cloned, making it difficult to study the specific interactions of these putative ligands with the HER-2/*neu* product. Subsequent identification, isolation and full purification of specific activators for p185$^{HER-2/neu}$ made possible the almost simultaneous cloning of two homologous ligands, one from human breast cancer cells, Heregulin (Holmes et al, 1992), and the other from ras-transformed rat fibroblasts, *neu* differentiation factor (Peles et al., 1992; Wen et al., 1992). Despite the different origin of heregulin (HRG) and *neu* differentiation factor

(NDF), the proteins encoded by these genes are identical at the amino acid level. More recent data indicate that both proteins induce phosphorylation of $p185^{HER-2/neu}$ as a result of either binding to this receptor with low affinity or to HER-2/HER-3 receptor heterodimers with a much higher affinity (Sliwkowski et al., 1994). Heregulin can also bind $p180^{HER-4}$ and induce its phosphorylation (Plowman, et al., 1993). Formation of HER-2/HER-4 receptor heterodimers has also been found (Karunagaran, et al., 1996). These as well as other results, including downregulation of binding of EGF to EGFR by Heregulin/NDF (Karunagaran, et al., 1995), suggest ligand binding can induce a series of complex interactions, resulting from heterodimerization and/or through transphosphorylation of members of class I RTKs. The mechanisms of type I receptor homo or heterodimerization occurring as a result of HRG binding to human breast as well as other epithelial and neural cells is currently the subject of intense research. There is now evolving consensus in the literature suggesting that the HER-2/*neu* receptor plays a critical role in heregulin signal transduction (Carraway III et al., 1995; Marikovsky et al., 1995; Pinkas-Kramarski, 1996).

A number of diverse and at time contradictory biological activities of Heregulin/NDF have been reported. Heregulin has been reported to have a growth stimulatory effect on a HER-2/*neu* overexpressing cell line SK-BR-3 (Holmes et al., 1992), as well as the HC11 mammary epithelial cells (Marte et al., 1995), mouse fibroblasts transfected with HER-2/*neu* and HER-3 (Carraway et al., 1995) and 32D murine hematopoietic cells transfected with different RTK I combinations (Pinkas-Kramarski, 1996).

The approach chosen in these studies to evaluate the biological function of heregulin involved the use of different cell growth assays and to employ a variety of human cell lines of epithelial origin with various levels of HER-2/*neu* expression. This was done to circumvent the possibility that any observed effect was due to unique characteristics of an individual cell line or assay technique, as well as to determine if there were generalized biological responses associated with this ligand-receptor system.

Evaluation of HRG activity as a growth factor on a series of human breast and ovarian cell lines (both non-malignant and malignant) expressing different levels of HER-2/*neu* was performed using two different assays. In the first assay, the total number of cells per well was quantified after a long term (10 days) treatment of cells with HRG. Cells were plated at low density, and after cell adherence to the plastic were treated with two different doses of HRG (1 and 10 nM). For the second approach, cells were plated at high density for a two day treatment with the same two doses of HRG. The surrogate measure of mitogenic activity was examined by measuring the incorporation of ^{3}H-thymidine into cellular DNA.

Cell counts

Normal mammary epithelial cells HMEC RV/NEO, as well as immortalized parental cells HBL-100 and the mock transfectants HBL-100 RV/NEO do not respond to HRG addition. However, after overexpression of HER-2/*neu* in the same cell lines (HMEC RV/H2 and HBL-100 RV/H2a) a dose-dependent response to HRG was observed. A concentration-dependent, growth stimulatory effect was obtained when breast tumor cells overexpressing HER-2/*neu* were treated with HRG. It is interesting to note that two adenocarcinoma breast cell lines expressing a single copy of HER-2/*neu* respond differently to HRG. MCF-7 parental cells and their mock transfected derivatives MCF-7

RV/NEO show a dose-related response to HRG. On the other hand, MDA-MB-231 cells transfected with the same control vector are non-responsive to HRG or respond minimally. The results of the proliferation experiments show the effects of a 7 kD recombinant form of HRG-b1, previously demostrated to have identical effects on $p185^{HER-2/neu}$ phosphorylation as the 45 kD ligand originally purified from the conditioned medium by MDA-MB-231 cells (Holmes, et al., 1992). When we used a full length recombinant form of HRG-b1 (FL-HRG-b1) to evaluate the effects of both recombinant forms of the ligand on the proliferation of MCF-7 cells parental similar results were seen. Results show a similar trend and show that after 10 days of incubation both forms of the recombinant ligand, the short 7 kD consisting of the EGF domain and the full length rHRG-b1, have equally potent stimulatory effects on the growth of the MCF-7 cell line, validating the use of the 7 kD recombinant form of the ligand in the previous experiments and thereafter.

^{3}H-thymidine incorporation

No significant differences between control-treated and HRG-treated cells, for both the breast and ovarian cell lines, were observed overall in this short-term ^{3}H-thymidine incorporation assay as compared to the longer term *in vitro* growth assays. Exceptions to this were the mock transfectants MCF-7 RV/NEO and the HER-2/*neu* overexpressors SK-BR-3, which showed dose-responsiveness to HRG treatment . The immortalized breast cell line HBL-100 transfected to overexpress HER-2/*neu* responded significantly to the highest concentration of HRG. The bulk of the results obtained in the ^{3}H-thymidine assay, which is a surrogate measurement of cell proliferation, and the actual quantitation of cell proliferation by direct cell counts, suggest a higher potency for HRG on long-term treatments as compared to this short-term assay. Confirmation of this hypothesis was obtained by quantitating the effects of both 7 kD HRG-b1 and FL-rHRG-b1 on a time-based cell proliferation assay (cell counts). Measurable effects of both forms of the ligands applied at 1 nM and 10 nM on MCF-7 parental cells and MCF-7 RV/H2 transfectants, started after 5 days of incubation . Minimal or non-measurable effects were observed after three days of treatment, validating the results of the ^{3}H-thymidine assay.

Two different approaches were then used to investigate the effects of HRG on the anchorage-dependent and anchorage-independent growth of the same panel of human breast and ovarian cell lines. Clonogenic assays evaluate the anchorage-dependent capacity of cells to establish and form colonies when plated on plastic at low densities, and provide an indication of aggressiveness of cell growth. Conversely, soft agar assays measure the anchorage-independent growth of cells in a semi-solid medium. Anchorage-independent growth is traditionally associated with acquisition of a transformed phenotype (Paraskeva, et al., 1990).

Clonogenicity

Analogous to the results obtained when measuring cell growth , the immortalized breast cell line HBL-100 only responded to HRG treatment in the clonogenic assay when overexpressing HER-2/*neu*.. Cells exhibited a dose-related response to HRG only after HER-2/*neu* transfection, and overexpression. Interestingly, cell lines that did not respond to treatment with the ligand at any dose in the cell growth assays, were responsive to treatment with HRG at both 1 and 10 nM in the clonogenic assays. The

effect of HRG at the two concentrations used to treat the breast low HER-2/*neu* expressors was variable. Again, a statistically significant consistent dose-response was obtained by treating high HER-2/*neu* expressor cell lines.

Growth in soft agar

Long term exposure of cell lines to HRG in a semi-solid medium demonstrated the capacity of this ligand to both promote and potentiate *in vitro* colony growth in soft agar of cells expressing HER-2/*neu* . HRG showed increased activity on the immortalized cell line HBL-100 transfected with HER-2/*neu* . All breast and ovarian tumor cell lines overexpressing HER-2/*neu* tested for colony formation in the semi-solid medium showed dose-response dependent colony formation (with the exception of the normal epithelial cell line HMEC RV/NEO, which did not respond to ligand addition even when engineered to overexpress HER-2/*neu,* results not shown here). HRG potentiates the capacity for anchorage-independent growth of the transformed breast cell lines MCF-7 RV/NEO, and MCF-7 RV/H2, as well as that of SK-BR-3 cells. It is interesting to note that HRG seemed to downregulate the amount of colonies formed by the MCF-7 RV/H2 cells when added at 10 nM compared to the 1 nM treatment with the ligand. We should stress here that this result does not implies an inhibitory effect of the ligand when used at 10 nM, because the number of colonies counted was still above those obtained counting the respective control-treated wells. Noteworthy, MCF-7 RV/H2 cells formed larger colonies when a 10 nM concentration of heregulin was used.

It is important to note that we used a 1 % concentration of fetal bovine serum in all four *in vitro* assays. This is a relevant point to keep in mind when comparing our results with other related reports in the literature in which the percentage of FBS in the growth medium is 10 or higher. High concentrations of FBS contain a great deal of growth factors (Sporn & Roberts, 1988; Vubricevic, et al., 1992) that could either mask, or some how modulate the activity we want to asses, in the case HRG activity.

The bulk of the results obtained after treatment of this panel of breast cell lines with two different doses of HRG, using 4 different *in vitro* cell growth assays, indicate a cell growth-stimulatory, rather than a growth-inhibitory role for this $p185^{HER-2/neu}$ activator in the conditions tested. Our results support the initial report by Holmes, et al (1992) indicating the mitogenic activity of both HRG-a and HRG-b1 on cell lines overexpressing HER-2/*neu*. Although we only tested recombinant b1 forms in the assays described here, our results show a consistent pattern of growth stimulatory effects. Cell cycle analysis were also performed and no cell cycle arrest was observed as a result of HRG treatment. Similar results for the growth-stimulatory activity of HRG tested on different conditions were recently reported (Lewis, et al., 1996). Under the conditions HRG was tested we, as well as others, have obtained no indication of growth-inhibitory and/or differentiation effects for heregulin, as reported by investigators working with NDF (Peles, et al., 1992; Bacus, et al., 1993). The effects of the monoclonal antibody antagonist 4D5 show a complete reversal of the growth inhibitory effects of HER-2*neu* overexpression of HRG treatment. These studies show that the response to 4D5 results in growth characteristics similar to the parental non-overexpressing cell lines.

Specific Aim III - To assess the biologic effects of the ligands and antibody alone and in combination on HER-2/*neu* expressing human breast cancer cells growing *in vivo.*

The last 12 months of funding we have been able to successfully expand and complete studies on the tumorigenicity of HER-2/*neu* transfected MCF-7 cells in response to heregulin and the 4D5 monoclonal antibody.

The main goal of this specific aim was the investigation of the biological effects of heregulin, both *in vitro* and *in vivo*, on human epithelial cells expressing different levels of the HER-2/*neu* proto-oncogene. Results presented so far correspond to the effects of heregulin on different cell growth assays performed in our laboratory *in vitro*. Next we describe for the first time in the literature the results obtained when a panel of cell lines, comprising single-copy level expressors to overexpressors for HER-2/*neu*, were used as reagents to study the effects of heregulin on cells growing *in vivo*. The results we obtained after treatment with heregulin of cells growing in nude mice support the role of this ligand as a growth-stimulatory factor.

Several types of assays have been used to study tumor progression (Paraskeva, et al., 1990). Tumorigenesis in the nude mice has been used, and is probably the best criteria known so far, to evaluate the changes in growth rate and differentiation promoted by diverse compounds in cells growing *in vivo* (Freshney, 1985).

The tumorigenic activity of HRG *in vivo* was evaluated by injecting breast and ovarian cells subcutaneously into female nude mice. In Figure 6 examples of the curves obtained for two of the breast cell lines tested are provided. When the estrogen-dependent breast cancer cell line MCF-7 was injected into nude mice bearing subcutaneously implanted estrogen pellets and then treated after tumor formation occurred, tumors exposed to rHRG-b1 increased in size over time, while those mice injected with the excipient control had progressively smaller tumors. HRG stimulation of the growth of the estrogen-dependent breast tumor cell line MCF-7 parental was 1.6-2 times over the growth of control-treated tumors by 15 days. On the other hand MCF-7 cells, transfected in our laboratory to overexpress HER-2/*neu,* formed considerable tumors in both groups. Again, heregulin treatment of mice injected with MCF-7 RV/H2 cells increased the tumor volume over the control treated group, achieving tumor sizes that were almost double when compared to those in the control-treated group by the end of the experiment.

Analysis of the p values shows dramatic differences for the growth of estrogen-dependent MCF-7 breast tumor cells in ovarectomized female nude mice. HRG itself was able to support the growth of MCF-7 parental cells as tumors without any kind of estrogen supplement. The growth of SK-BR-3 tumors in the nude mice was also significantly different from the control-treated group in mice treated with HRG. However, tumors obtained were too small (approximately 30 cubic millimeters) to be considered of biological meaning. A statistically significant difference, as indicated by a p value of less than 0.01, was obtained for the growth of the immortalized breast cell line HBL-100 after HRG treatment of mice injected with both the mock- and the HER-2/*neu* -transfectants. Nevertheless, the tumors did not grow to a significant size subtracting to the statistical value. The lack of a meaningful difference for the growth of MCF-7 RV/H2 transfected cells in non-ovarectomized nude mice treated with HRG, compared to the control treated tumors, is due to the ability of the HER-2/*neu* overexpressors to grow to a similar extent as the ligand-treated tumors. In mice

injected with MCF-7 RV/H2 cells the tumor volume was significantly higher in the HRG-treated group the last week of the experiment, compared to tumor sizes in the control-treated group. However the differences were not statistically significant over the course of the experiment. Overexpression of HER-2/*neu* itself has been reported to confer growth advantage (Hudziak, et al., 1987; Chazin, et al., 1992).

In summary, the results of these *in vivo* experiments further support the role of heregulin as a growth-stimulatory, rather than a growth-inhibitory factor. In general our results do not support a role for heregulin on breast cancer cell differentiation. It is generally found that a cell line exhibits increased clonogenicity, and anchorage independent growth (measured as growth in a semi-solid medium) before it becomes tumorigenic in athymic nude mice. Differentiation of tumors correlates with reduced malignancy, resulting in a decrease in tumorigenicity (Toth, 1985). In other words, conditions which induce differentiation in rat mammary carcinoma also reduce tumor growth *in vivo* (Fresney, 1985). The possible effects of heregulin on cell differentiation, as claimed by Peles, et al (1992), are still controversial and will require more detailed investigation. The mechanism by which heregulin is able to overcome MCF-7 parental breast cells growth-dependence on estrogen is a matter of ongoing research (Matsuda et al, 1993; Pietras et al., 1995). The 4D5 studies presented in the preliminary data of the initial proposal have again been repeated and confirmed in the past months and clearly demonstrate the growth inhibitory effects of the monoclonal antibody at dosages which maintain a serum level of 15-20 mg/ml which is a level that is achievable clinically.

Specific Aim IV - To assess the biologic effects of an additional molecular alteration i.e. mutation of the p53 gene in combination with HER-2/*neu* overexpression in human breast cells both *in vitro* and *in vivo*

We have had problems achieving the goals of this specific aim due to difficulties with the p53 vectors. These difficulties seem center around the efficiency of transfection of the human breast epithelial (normal and malignant) cell lines with our constructs. To date we have only tried the retroviral-based vectors. Our plan is to attempt to use adenoviral based vectors carrying the mutant p53 gene if additional attempts with the retroviral vectors are not productive. We still believe that this is an important undertaking in that it should provide some insights into the potential interaction of alterations on HER-2/*neu* and p53, specifically in normal breast epithelial cells. If the retroviral vectors continue to be ineffective, it will be worth the additional effort to use the adenovirus system

CONCLUSIONS

Based on the data obtained during the past 12 months of funding for the proposal 8/95-7/96, we have developed information which continues to clarify issues regarding the role of the HER-2/*neu* gene in human breast cancer. This information includes:

A. Overexpression of the HER-2/*neu* gene in human breast cancer cells has direct effects on the biologic behavior of these cells which indicate that the alteration has a direct pathogenetic role in the behavior of HER-2/*neu* overexpressing breast cancer.

This is a critical observation if the alteration is to be targeted in new therapeutic approaches. We have also addressed the issue of chemotherapeutic drug sensitivity and resistance on HER-2/*neu* overexpressing cells again developing information of potential use clinically.

B. We have exhaustively studied the effects of the heregulin ligand on HER-2/*neu* overexpressing and non-overexpressing cells and demonstrate that it is clearly a growth stimulatory molecule in *in vitro* studies. We have again demonstrated and further studies the *in vitro* growth inhibitory effects of the 4D5 antibody demonstrating its potential use clinically based on these *in vitro* preclinical data.

C. The *in vivo* data developed with heregulin and 4D5 mimic the effects we see *in vitro* and further underscore the clinical potential of the monoclonal antibody in preclinical studies. We feel that these data will allow us to gain insight into how we might optimally utilize this antibody clinically.

D. The initial results with the p53 retroviral vectors have been disappointing but this is due to technical difficulties rather than non-relevant biologic results. We feel that this question is still of interest if we can successfully introduce the mutant p53 gene into HER-2/*neu* overexpressing breast cancer cells. As a result, we will continue to pursue this specific aim.